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Characterization of Glutamine-Requiring Mutants of *Pseudomonas aeruginosa*

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Revertants were isolated from a glutamine-requiring mutant of *Pseudomonas aeruginosa* PAO. One strain showed thermosensitive glutamine requirement and formed thermolabile glutamine synthetase, suggesting the presence of a mutation in the structural gene for glutamine synthetase. The mutation conferring glutamine auxotrophy was subsequently mapped and found to be located at about 15 min on the chromosomal map, close to and before *hisII4*. Furthermore, in transduction experiments, it appeared to be very closely linked to *gln-2022*, a suppressor mutation affecting nitrogen control. With immunological techniques, it could be demonstrated that the glutamine auxotrophs form an inactive glutamine synthetase protein which is regulated by glutamine or a product derived from it in a way similar to other nitrogen-controlled proteins.

Nitrogen control, that is, regulation of enzyme formation by the availability of ammonia, has been demonstrated for a number of enzymes in *Pseudomonas aeruginosa*. It includes enzymes involved in the utilization of urea (17), histidine (21, 27), arginine (25), acetamide (16), nitrate reductase (31), and also proteins that are responsible for the formation of glutamate and glutamine (2, 15, 17). We have shown previously that glutamine or some compound derived from it plays a major role in the regulation of proteins that are subject to nitrogen control (15). This conclusion was based on the observation that glutamine synthetase-negative mutants were impaired in the repression of urease and histidase by excess ammonia, whereas NADP-dependent glutamate dehydrogenase was not elevated. Only growth with excess glutamine, which could be obtained in a mutant with reduced conversion of glutamine, caused repression of urease and histidase and derepression of NADP-dependent glutamate dehydrogenase synthesis.

We have also obtained mutants from *Pseudomonas aeruginosa* that show disturbed nitrogen control (14). These mutants could not utilize a number of amino acids and did not show derepression of urease and glutamine synthetase formation under nitrogen limitation, whereas NADP-dependent glutamate dehydrogenase was not repressed. Suppression of this phenotype by mutation at another chromosomal site was observed, and both mutations were mapped on the chromosome.

In enteric bacteria, there are at least three genes claimed to be involved in nitrogen control. The *glnF* gene, whose product is unknown, was found to be required for glutamine synthetase production and proper derepression of other proteins subject to nitrogen control (7, 8). The *glnB* gene encodes the P_{II} regulatory protein in the glutamine synthetase adenylylation system and has been reported to be required for glutamine synthetase derepression (6, 29). Finally, the presence of a regulatory gene, called *glnG* (26), *glnR* (19), or *ntrC* (24, 28) and located close to the structural gene for glutamine synthetase, *glnA*, has been demonstrated. Mutations in this regulatory gene caused a loss of the ability to derepress glutamine synthetase and other nitrogen-controlled proteins. They were also obtained as suppressors from mutations in *glnF* (19, 24). Recently, the product of *glnG* has been identified as a 55,000-dalton protein (1, 24). Probably, *glnG* (1) and *ntrC* (24) are separated from *glnA* by a third gene which also can harbor mutations that affect nitrogen control. The product of this gene is a 36,000-dalton protein (24). The two regulatory genes and the glutamine synthetase structural gene were found to be part of one operon, transcribed in the direction from *glnA* to *glnG* (1).

It is completely unknown whether the mechanism for nitrogen control in *P. aeruginosa* has similarities to the system of enteric bacteria. In this paper, we present some properties of glutamine synthetase-negative mutants that may be relevant to the understanding of nitrogen control in *P. aeruginosa*.

MATERIALS AND METHODS

Organisms. All bacterial strains used are derivatives of *P. aeruginosa* PAO1 (Table 1). Strain PAO2175 (23) was the wild-type strain from which the glutamine synthetase-negative mutants PAO4501 and PAO4506 (formerly PAO4001 and PAO4006, respectively), were derived (15). Strains. plasmids, and phages for genetic experiments were kindly donated by B. Holloway (Monash University, Clayton, Australia) and D. Haas (ETH, Zurich, Switzerland).

Growth media. Liquid synthetic media contained (per liter): 4.3 g of $Na_2HPO_4 \cdot 2H_2O$, 2.2 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, and 1.8 mg of $FeSO_4 \cdot 7H_2O$. Trisodium citrate $\cdot 2H_2O$ (1%) was used as the carbon source, and a nitrogen source was added as indicated. The pH after sterilization was 7.0.

For solid media, the minimal medium of Vogel and Bonner (33) was used. Amino acids were added at 1 mM when necessary, except glutamine, which was used at 0.2%. Glutamine solutions were always prepared freshly and filter sterilized.

Growth conditions. For experiments in which enzyme formation was studied, growth media were inoculated with washed and diluted precultures on nutrient broth plus glutamine. Cultures were grown overnight at 37°C and harvested at an optical density at 600 nm of 0.3 to 0.6. Glutamine limitation was achieved by adding glutamine at a growth-limiting rate as described previously (15).

Isolation of revertants. Revertants from strain PAO4501 were obtained after mutagenesis. A preculture of PAO4501 on nutrient broth plus glutamine was divided into 40 1-ml portions and treated with 10 μl of ethylmethane sulfonate per ml for 1 h at 37°C without shaking. After overnight growth on nutrient broth plus glutamine, samples were spread on nutrient broth plates and incubated at 30°C. Revertants were picked off after 3 days and purified on the same medium. Twenty-four independent revertants were obtained.

Enzyme assays. Glutamine synthetase activities were estimated in crude extracts prepared by sonication in IMMK buffer (10 mM imidazole-hydrochloride [pH 7.1], 2 mM $MnCl_2$, 1 mM β-mercaptoethanol, and 100 mM KCl). The assays were carried out at pH 7.9 as described previously (15, 17). Glutaminase was measured in crude extracts prepared in 10 mM Tris-hydrochloride (pH 7.2) containing 100 mM KCl. Activities were measured by following the conversion of γ-glutamylhydroxamate according to Brown and Tata (3). Protein concentrations were measured by the method of Lowry et al. (22), using bovine serum albumin as a standard.

Genetic techniques. In conjugation experiments, R68.45 was used as the chromosome-mobilizing plasmid (10). The construction of donor strains carrying R68.45 was done as described by Haas and Holloway (10). Plate matings were carried out by the method of Stanisich and Holloway (32). Desired strains were constructed with R68.45-mediated conjugations as described by Haas and Holloway (10).

For transduction experiments, phage suspensions were prepared by the soft-agar layer method. Transductions were performed by the method of Haas et al. (11). The procedure for the prototroph reduction transduction test was described by Fargie and Holloway (5).

TABLE 1. Strains of *P. aeruginosa*

Strain	Genotype	Comments (reference)
PAO222	*ilvB/C226 hisII4 lys-12 trp-6 met-28 proA82*	(9)
PAO303	*argB18*	(13)
PAO2175	*met-9020 catA1*	(23)
PAO4501	*met-9020 catA1 glnA2001*	(15)
PAO4502	*met-9020 catA1 glnA2002*	(15)
PAO4503	*met-9020 catA1 glnA2003*	(15)
PAO4504	*met-9020 catA1 glnA2004*	(15)
PAO4505	*met-9020 catA1 glnA2005*	(15)
PAO4506	*met-9020 catA1 glnA2006*	(15)
PAO4508	*met-9020 catA1 glnA2008*	Revertant of PAO4501
PAO4510	*ilvB/C226 hisIV59 lys-12*	(14)
PAO4516	*ilvB/C226 hisII4 lys-12 trp-6 proA82*	Met^+ transductant of PAO222 × F116L (PAO1)
PAO4519	*ilvB/C226 hisII4 lys-12 trp-6*	(18)
PAO4522	*met-9020 catA1 gln-2020 gln-2022*	$GlnR^c$ phenotype (17)
PAO4550	*leu-8 glnA2001*	(16)
PAO4551	*glnA2001*	Arg^+ Gln^- recombinant of PAO303 × PAO4501(R68.45)

Transconjugants and transductants obtained in mapping experiments were tested by replica plating for auxotrophic markers. The $GlnR^c$ phenotype, which is characterized by derepressed urease and glutamine synthetase syntheses in the presence of ammonia, was tested with a urease spot assay as described previously (14). Thermosensitive glutamine auxotrophy was determined by testing the growth on plates containing no glutamine at 30 and 42°C.

Purification of glutamine synthetase. Glutamine synthetase was purified from strain PAO2175 grown on citrate medium supplemented with 0.2% KNO_3 as a nitrogen source. Crude extract was prepared by sonication (15) in IMMK buffer, which was also used during the isolation of the enzyme. The extract was treated with streptomycin sulfate (1%), and the precipitate was removed by centrifugation. After dialysis, the extract was subjected to heat treatment for 15 min at 65°C, and the precipitate formed was removed by centrifugation. The increase in total activity during these two steps may be caused by removal of compounds that have an inhibitory effect on glutamine synthetase activity. Upon fractionation with a saturated $(NH_4)_2SO_4$ solution, glutamine synthetase activity was found in the 50 to 70% saturation precipitate. After dialysis of the dissolved enzyme, it was absorbed on a DEAE-cellulose DE52 column (1.4 by 2

cm) and eluted with a linear gradient of 200 ml of 0 to 1 M KCl in IMMK buffer. The most active fractions were dialyzed and purified further on Affigel Blue as described by Lepo et al. (20). The purification scheme is summarized in Table 2. The resulting protein preparation was used for the generation of antibodies. It showed one protein band after polyacrylamide gel electrophoresis of a 50-μg sample. By using an activity strain for glutamine synthetase, one active band with the same electrophoretic mobility was found.

Immunological techniques. Antibodies against glutamine synthetase were prepared in white New Zealand rabbits. The first injection contained 300 μg of protein in Freund complete adjuvant. Four subsequent injections, given at 1-week intervals, contained 150 μg of protein each and were given in Freund incomplete adjuvant. One week after the last injection, the animals were bled and antiserum was prepared.

Ouchterlony immunodiffusion revealed that the crude antiserum was not completely specific for glutamine synthetase. Both the crude serum and control serum produced a precipitin band with a protein that was immunologically different from glutamine synthetase. The specificity of the serum was improved by treatment with crude extract from strain PAO2175 containing a low level of glutamine synthetase protein. Therefore, crude serum was mixed with extract from strain PAO2175 grown on citrate medium supplemented with ammonia and glutamine. After 1 h at 37°C, the precipitate was removed by centrifugation, and the resulting specific serum was used for immunological experiments.

The presence of inactive glutamine synthetase protein in extracts from glutamine synthetase-negative mutants was determined with the quantitative inhibition method (4). In this assay, the level of inactive protein in an extract can be estimated by measuring the amount of serum neutralized by a known quantity of extract. The remaining amount of antibody is quantitated by adding known and sufficient amounts of active glutamine synthetase, so that an excess is obtained, and by the subsequent determination of residual enzyme activity. The procedure followed was essentially that of Kaminskas et al. (18). At the equivalence point, the titer of the serum was 3.5 U of glutamine synthetase precipitated per ml of antiserum. One unit of inactive glutamine synthetase protein is defined as the amount that inactivates the same quantity of serum as does 1 U of enzymatically active glutamine synthetase.

TABLE 2. Purification of glutamine synthetase from *P. aeruginosa*

Purification step	Vol (ml)	Amt of protein (mg)	Activity (U)	Sp act (U/mg of protein)	% Yield
Crude extract	25	380	157	0.41	100
Streptomycin supernatant	25	360	163	0.45	104
Heat treatment	25	133	192	1.45	122
$(NH_4)_2SO_4$ precipitation	8	69	138	2.0	88
DEAE-cellulose	13	15	121	8.1	77
Affigel Blue	6	5.5	99	18	63

RESULTS

Revertants from strain PAO4501. We have previously described the isolation of glutamine-requiring mutants of *P. aeruginosa* (15). Because the loss of glutamine synthetase activity in at least two of these strains caused altered regulation of the synthesis of a number of proteins subject to nitrogen control, it was important to determine whether the genetic defect is located in the structural gene for glutamine synthetase or in some gene with a regulatory function. Therefore, revertants were isolated from strain PAO4501 and tested for their regulatory properties and thermosensitivity. Of 24 independently isolated revertants, one strain was able to grow on solid medium in the absence of glutamine at 30°C but not at 42°C. When the medium was supplemented with glutamine, growth occurred at both temperatures. The temperature-sensitive glutamine auxotrophic revertant was designated PAO4508. All revertants isolated showed normal derepression of urease on plates with nitrate and repression of urease on plates with ammonia as nitrogen source, as could be demonstrated with the spot test for urease activity described previously (14).

Heat lability. The possibility that the temperature sensitivity of strain PAO4508 was due to increased heat lability of glutamine synthetase was examined. Crude extracts from strains PAO4508 and PAO2175 were heated at 62°C, and the course of glutamine synthetase inactivation was followed (Fig. 1). It appeared that glutamine synthetase activity in extracts prepared from strain PAO4508 was inactivated much more rapidly than the enzyme in extracts from the wild-type strain PAO2175. The increased heat lability of glutamine synthetase in strain PAO4508 indicates the presence of a mutation in the structural gene for glutamine synthetase in this strain. Since strain PAO4508 was isolated as a revertant from strain PAO4501, this result also suggests the presence of a mutation in the structural gene for glutamine synthetase in strain PAO4501.

Genetic mapping of *gln* mutations. Plasmid R68.45-mediated conjugations with multiple marked donor strains were used to obtain a map position for the mutation conferring a glutamine requirement in strain PAO4501. Initial crosses indicated that this locus, *glnA2001*, is located in the early region of the chromosome, somewhere in the 10- to 20-min region. Crosses between strains PAO222(R68.45) and PAO4501 as the acceptor revealed that *glnA2001* is located close to and before *hisII4* at 16 min (Table 3). Linkage between *glnA2001* and *hisII4* was 72%, and linkage between *glnA2001* and *lys-12* was 34%. Recombinants with the phenotype Gln^+ His^+

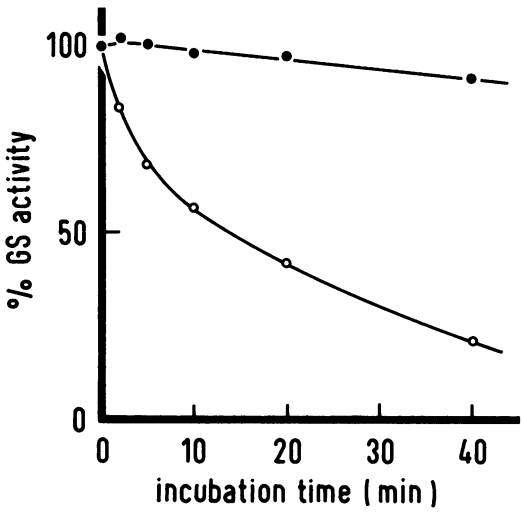

FIG. 1. Thermolability of glutamine synthetase from strain PAO4508. Cells from strains PAO2175 (●) and PAO4508 (○) were grown in citrate medium under glutamine limitation, and crude extracts were prepared in IMMK buffer. The extracts were treated at 62°C, and at different time intervals samples were withdrawn, centrifuged, and assayed for residual glutamine synthetase activity in the supernatant. The initial activities of the extracts were 451 mU/ml (1.5 mg of protein per ml) and 248 mU/ml (1.6 mg of protein per ml) for strains PAO2175 and PAO4508, respectively.

Lys$^-$ were not found, suggesting that four crossovers were required to obtain these strains.

The mutation conferring glutamine auxotrophy in strain PAO4506, *glnA2006*, was mapped with similar crosses: PAO222(R68.45) × PAO4506. The results again suggested a map position close to and before *hisII4*. Linkage values of *glnA2006* and *hisII4* were 74 and 82%, dependent on the use of *ilvB/C226* or *proA82*, respectively, as the contraselective marker (Table 3).

The mutation that caused the formation of thermolabile glutamine synthetase in strain PAO4508 was also located close to *hisII4*. Strain PAO4508 could not be used as an acceptor in genetic experiments because its reversion rate is too high. However, when it was used as a donor with strain PAO4516 as the recipient, 84% of the His$^+$ recombinants obtained showed thermosensitive glutamine auxotrophy (Table 3).

Transductions. The strong linkage between *glnA2001* and *hisII4* in conjugations suggested that these mutations could be cotransducible. This possibility was tested with the generalized transducing phage F116L. Because F116L is not able to effectively propagate on or transduce strain PAO2175 and its derivatives, recombinants having the *glnA2001* mutation in another genetic background were used as recipients. Cotransduction values of *hisII4* with *glnA2001* of 15 and 18% were obtained with strains PAO4550 and PAO4551, respectively, as the recipients (Table 4). The linkage of these markers was also tested with phage G101, which can grow and the transduce strain PAO2175-derived strains. A value of 6% cotransduction was obtained with strain PAO4501 as the recipient (Table 4).

Recently, we described the presence of a suppressor mutation, *gln-2022*, that relieves the inability of certain regulatory mutants from strain PAO2175 to derepress some enzymes

TABLE 3. Genetic mapping of *glnA*[a]

Strain		Marker		No. of conjugants scored	Recombinant phenotype	% Frequency	No. of crossovers required
Donor	Recipient	Selected	Contraselected				
PAO222(R68.45)	PAO4501	*glnA2001*$^+$	*ilvB/C226*	210	Gln$^+$ His$^+$ Lys$^+$	28	2
					Gln$^+$ His$^-$ Lys$^+$	38	2
					Gln$^+$ His$^-$ Lys$^-$	34	2
					Gln$^+$ His$^+$ Lys$^-$	0	4
PAO222(R68.45)	PAO4506	*glnA2006*$^+$	*ilvB/C226*	131	Gln$^+$ His$^+$ Lys$^+$	26	2
					Gln$^+$ His$^-$ Lys$^+$	39	2
					Gln$^+$ His$^-$ Lys$^-$	35	2
					Gln$^+$ His$^+$ Lys$^-$	0	4
PAO222(R68.45)	PAO4506	*glnA2006*$^+$	*proA82*	205	Gln$^+$ His$^+$ Lys$^+$	18	2
					Gln$^+$ His$^-$ Lys$^+$	43	2
					Gln$^+$ His$^-$ Lys$^-$	39	2
					Gln$^+$ His$^+$ Lys$^-$	0	4
PAO4508(R68.45)	PAO4516	*hisII4*$^+$	*met-9020*	84	His$^+$ Gln$^+$	16	2
					His$^+$ Gln^{TS} [b]	84	2

[a] The results suggest the order *glnA hisII4 lys-12*.
[b] TS, Temperature sensitive.

TABLE 4. Transduction of *gln* mutations

Phage	Recipient	Marker selected	No. of transductants scored	Phenotype of transductants	% Frequency
F116L (PAO222)	PAO4550	*glnA2001*$^+$	121	Gln$^+$ His$^+$	85
				Gln$^+$ His$^-$	15
F116L (PAO222)	PAO4551	*glnA2001*$^+$	191	Gln$^+$ His$^+$	82
				Gln$^+$ His$^-$	18
G101 (PAO4519)	PAO4501	*glnA2001*$^+$	111	Gln$^+$ His$^+$	94
				Gln$^+$ His$^-$	6
G101 (PAO4522)	PAO4501	*glnA2001*$^+$	235	Gln$^+$ GlnR$^+$	1
				Gln$^+$ GlnR^c	99
G101 (PAO4522)	PAO4506	*glnA2006*$^+$	194	Gln$^+$ GlnR$^+$	5
				Gln$^+$ GlnR^c	95
G101 (PAO4522)	PAO4519	*hisII4*$^+$	190	His$^+$ GlnR$^+$	91
				His$^+$ GlnR^c	9

subject to nitrogen control (14). This suppressor mutation caused high-level synthesis of urease and glutamine synthetase, even when excess ammonia was present in the growth medium. Mapping experiments demonstrated that it was located close to and before *hisII4*, just as the *glnA2001* locus described here. When the cotransduction of *gln-2022* with *glnA2001* and *glnA2006* was tested with phage G101, the results showed 99 and 95% linkage of these markers, respectively (Table 4). Also, *gln-2022* and *hisII4* appeared to be cotransducible (Table 4).

Previously, six independent glutamine auxotrophs were obtained (15). Five of these strains did not form detectable glutamine synthetase activity, whereas one strain, PAO4505, formed a low amount of glutamine synthetase and appeared to be leaky on rich medium (15). On the basis of tranductional analysis, all *gln* mutations were very closely linked on the chromosome. Gln$^+$ transductants were not found in crosses when one of the Gln$^-$ strains was used as the recipient and phage G101 grown on a Gln$^-$ mutant was used as the tranducing agent. The phage preparations used were able to produce His$^+$ transductants with strain PAO4510 as the recipient. Furthermore, phage G101 grown on strain PAO2175 yielded Gln$^+$ transductants when the Gln$^-$ mutants were used as recipients.

Glutamine requirement. When amino acid auxotrophic mutants of *P. aeruginosa* are grown in liquid cultures, high amounts of the respective amino acids are often required because the supplied compound is used for catabolic reactions rather than only for the fulfillment of the auxotrophic requirement. This was also the case with our glutamine auxotrophs. All five tight auxotrophs isolated previously required high amounts of glutamine when grown in batch culture. However, one strain, PAO4506, required even higher quantities of glutamine than the other mutants. With 0.2% glutamine in citrate-ammonia medium, the final densities of 0.14 and 0.6 mg (dry weight) per ml were obtained for strain PAO4506 and the other mutants, respectively (16). Conjugational crosses with strain PAO4501 as the donor strain invariably produced glutamine auxotrophic recombinants with the higher glutamine requirement, just as was found when strain PAO4506 was used as the donor (data not shown). It follows that strain PAO4501 must have a second lesion that reduces glutamine conversion and saves more of the amino acid for use as a growth factor.

It was attempted to correlate the difference in glutamine requirement with glutaminase activities. In extracts from cells grown on citrate-ammonia medium supplemented with excess glutamine, the glutaminase activities were 220 and 70 mU/mg of protein for strains PAO4506 and PAO4501, respectively.

We have not yet been able to obtain a map position for the mutation that reduces the glutaminase activity. Its phenotype is clear only in a glutamine synthetase-negative background, where it can be tested by its effect on growth yield. Results from conjugations of strain PAO4501(R68.45) with strain PAO222 as the recipient indicate that the mutation is not located between *ilvB/C* at 7 min and *proA* at 42 min. All recombinants, also from repeated crosses in which the whole region between *ilvB/C* and *proA* was transferred, showed the high glutamine requirement.

Regulation of inactive glutamine synthetase. It

was not known whether the five mutants that contain no detectable glutamine synthetase activity produce an inactive glutamine synthetase protein. This question was relevant because the presence of an inactive protein would suggest that glutamine synthetase structure rather than formation is affected in the mutants. Furthermore, we wanted to investigate the regulation of the inactive protein, if it was formed.

With antiserum against purified glutamine synthetase, it was possible to demonstrate the presence of inactive glutamine synthetase protein in extracts from all mutants tested (Table 5). The inactive enzyme was present at even higher amounts than the enzyme in the wild-type strain when cells were cultivated under glutamine limitation. The results obtained with strain PAO4501 indicated that the formation of inactive glutamine synthetase was repressed only when the cells were cultivated with excess glutamine. In this respect, the formation of inactive glutamine synthetase is regulated in a way similar to urease. Both proteins are no longer repressed by ammonia and glutamate but only by excess glutamine in strain PAO4501 (15; Table 5). In strain PAO4506, the inactive protein and urease were not repressed during growth in the presence of excess glutamine.

DISCUSSION

The results presented in this paper indicate that five glutamine-requiring mutants isolated previously (15) have a defect in *glnA*, the structural gene for glutamine synthetase. The formation of thermolabile glutamine synthetase by a revertant from strain PAO4501 and the synthesis of inactive glutamine synthetase by the mutants provide strong evidence for a defect in glutamine synthetase structure rather than regulation. Transductional analysis showed that all mutations are strongly linked. A chromosomal location for *glnA* was obtained by R68.45-mediated conjugations. Three-factor crosses indicated that *glnA* was close to and before *hisII4* at 16 min, and linkage to this marker was confirmed by transductions with phages G101 and F116L (Fig. 2).

Previously, *gln-2022*, a suppressor mutation that affects nitrogen regulation, was mapped in the same region (14). This mutation suppressed the *gln-2020* mutation, which caused a loss of the ability to derepress urease and glutamine synthetase and repress NADP-dependent gluta-

TABLE 5. Formation of inactive glutamine synthetase

Strain	Growth medium[a]	Enzyme level (mU/mg)[b]	
		Inactive glutamine synthetase	Urease
PAO4501	Gln_1	250	2,100
PAO4501	Amm + Glu + Gln_1	530	4,100
PAO4501	Amm + Gln_e	44	100
PAO4502	Amm + Glu + Gln_1	168	480
PAO4503	Amm + Glu + Gln_1	990	3,400
PAO4504	Amm + Glu + Gln_1	865	3,600
PAO4506	Amm + Gln_e	310	3,400

[a] The growth medium contained 1% trisodium citrate · $2H_2O$ as a carbon source and a nitrogen source as indicated. Gln_1, Glutamine added at a growth-limiting rate; Amm, 0.2% $(NH_4)_2SO_4$; Glu, 0.2% glutamic acid; Gln_e, 1% glutamine.

[b] The levels of inactive glutamine synthetase and urease are expressed in mU/mg of protein (see text). Crude extract from the parent strain PAO2175 grown under glutamine limitation contained 120 mU of glutamine synthetase and 2,200 mU of urease per mg of protein.

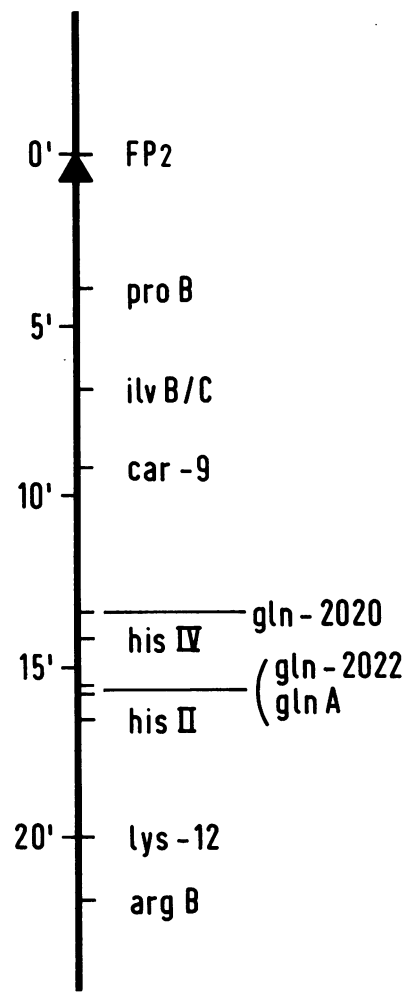

FIG. 2. Genetic map of *P. aeruginosa* PAO (12, 30). The mutations *proA82* and *met-9020* are located at 40 min and at about 60 min, respectively.

mate dehydrogenase synthesis during nitrogen-limited growth. The strong transductional linkage found here shows that *gln-2022* and *glnA* are located very close to each other on the chromosome. Conceivably, *gln-2020* and *gln-2022* are in regulatory genes, with functions similar to the enteric bacterial *glnF* and *glnG* (or *glnR*), respectively. Mutations in *glnG* could be isolated as suppressors of *glnF* (19, 24), a gene whose function is required for proper nitrogen control (7, 8). The *glnG* gene is located close to the structural gene for glutamine synthetase (19, 26), just as is *gln-2022*. Probably the *gln-2022* mutation is not located in the structural gene for glutamine synthetase. Strain PAO4522 showed thermosensitive growth with a number of poor nitrogen sources, e.g., nitrate, but not with ammonia, and we were not able to detect increased thermolability of glutamine synthetase. Fine-structure analysis and physical mapping will be required for the establishment of the precise location of the mutations and of gene orders.

The best characterized glutamine synthetase-negative strains, PAO4501 and PAO4506, have different regulatory properties. In PAO4501, derepression of a number of enzymes subject to nitrogen control, e.g., urease, histidase (15), and amidase (16) occurred during glutamine limitation, but not during growth with excess glutamine. Derepression of NADP-dependent glutamate dehydrogenase occurred only under conditions of excess glutamine (15). In strain PAO4506, proteins subject to nitrogen control were always present at high levels, even during growth with excess glutamine, and NADP-dependent glutamate dehydrogenase was always low (15). The synthesis of the inactive glutamine synthetase protein in the glutamine-requiring mutants was found to be regulated in a way similar to urease formation (Table 5). In strain PAO4501, repression occurred only during growth with excess glutamine, whereas in strain PAO4506, both urease and inactive glutamine synthetase levels remained high when the medium contained excess glutamine.

In strain PAO4506, glutamine was found to be subject to rapid degradation, and this was believed to explain the regulatory properties (15). The difference between strains PAO4501 and PAO4506 was not due to a different genetic basis for glutamine requirement or loss of glutamine synthetase activity. The mutations in both strains were found to map in the same chromosomal region (Table 3), and transductional analysis showed that *glnA2001* and *glnA2006* are very close. Strain PAO4501 was found to contain an additional mutation that reduces glutamine requirement and alters the regulatory properties. When *glnA2001* was transferred to another genetic background, the resulting Gln− strains showed regulatory properties similar to those of strain PAO4506 (data not shown) and a high glutamine requirement. The additional mutation in strain PAO4501 seems to reduce glutamine requirement by reducing glutaminase activity. Strain PAO4506 showed three- to four-fold higher glutaminase activities than strain PAO4501. It remains to be established how glutaminase activity is affected and where the mutation responsible is located on the genetic map.

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